

**ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES**



**ASSESSMENT OF BLOOD AGAR MEDIA FOR
THE DIAGNOSIS OF TUBERCULOSIS**

*A Thesis Submitted to the School of Graduate studies of Addis Ababa
University in Partial Fulfillment of the Requirements for the Degree of
Master of Science in Biotechnology*

**By
Wegene Tamene Mekasha**

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Abbreviations

AFB	Acid fast bacilli
AIDS	Acquired Immunodeficiency Syndrome
BCG	Bacillus Calmette Guerin
CD	Cluster of differentiation
CFU	Colony forming units
DOTS	Directly Observed Treatment short course
DST	Drug susceptibility testing
EHNRI	Ethiopian Health and Nutrition Research Institute
EMB	Ethambutol
HIV	Human Immunodeficiency Virus
IL-12	Interleukin 12
INF- γ	Interferon gamma
INH	Isoniazid
LJ	Lowenstein-Jensen media
LPA	Line probe assay
M.TB	<i>Mycobacterium tuberculosis</i>
MDR	Multi drug resistance
MGIT	Mycobacteria growth indicator tube
MODS	Microscopically Observed Drug Susceptibility
MTBC	<i>Mycobacterium tuberculosis</i> complex

NALC-NaOH N-Acetyl L-Cysteine Sodium hydroxide

NPV Negative predictive value

NTM Non *Mycobacterium tuberculosis* complex Mycobacteria

OADC Oleic acid, Albumin, Dextrase and Catalase

PANTA Polymixin B, Ampoteracin B, Nalidixic acid, Trimetoprim and Azulocilic

PBS Phosphate buffer solution

PPV Positive predictive value

PZA Pyrazinamide

RIF Rifampicin

SM Streptomycin

TB Tuberculosis

TNF- α Tumor necrosis factor Alpha

TTD Time to detection

WHO World Health Organization

Abstract

Diagnosing tuberculosis is a challenge because of the complex nature of the bacteria. Recently, the need for TB culture is growing due to the increasing demand for TB drug susceptibility testing (DST) and due to the high incidence of TB/HIV co-infection, which faces a great deal of misdiagnosis. Even though there are a number of better culture technology systems, most of these technologies are not feasible and affordable for resource limited countries like Ethiopia. Therefore, resource limited countries mainly rely on available solid culture system for *Mycobacterium* isolation and DST despite the fact, they are time consuming, costly and need stringent media preparation procedure. Thus, this study was aimed to assess the performance of 5% sheep blood agar media for the isolation and DST of *M. tuberculosis* complex. Blood agar is simple to prepare, readily available, and cheap media. 107 clinical specimens were collected from patients referred to EHNRI for TB culture. Specimens were liquefied and decontaminated by N-acetyl L-cysteine sodium hydroxide method and cultured on Lowenstein-Jensen media, blood agar media and BACTEC MGIT 960 system. Species identification was done using Capilla TB-Neo (TAUNS Laboratories Inc, Numazu, Japan). DST for *M. tuberculosis* complex isolates were performed using proportion method on LJ and blood agar media. From the total 107 specimens cultured, 2 specimens with persistent contamination were excluded from analysis, and analyses were done with the remaining 105 specimens. The sensitivity, specificity, PPV and NPV of blood agar media was 98, 98.2, 98 and 98.2%, respectively, as compared to LJ media, whereas the sensitivity, specificity, positive predictive value and negative predictive value of blood agar media were 87.2, 100, 100 and 87.2% respectively, when compared to MGIT. Mean time for culture positivity was 9.3, 17.3 and 22.7 days for MGIT, blood agar and LJ media, respectively and the difference was statistically significant ($P < 0.0001$). On the other hand, concordance between blood agar media and LJ media for DST was, 97.7% for Isonizid, 100% for rifampin, 90.7% for streptomycin and 97.7% for etambutol. The contamination rate was 5.1, 9.7 and 14.8% for blood agar media, LJ media and MGIT, respectively. In conclusion, blood agar media was correlated well with LJ media both for the isolation and drug susceptibility testing of *M.TB* and it was faster than LJ media.

Keywords: Blood agar media, Diagnosis, Drug susceptibility testing, Isolation, Tuberculosis

1. Introduction

1.1. Epidemiology of Tuberculosis

Despite the efforts put on it, TB remains to be one of the major public health problems in the world. In 2007 alone, 9.27 million estimates of new TB cases occurred worldwide (WHO, 2009a). Among them, 4.1million (44%) were new smear positive cases. India, China, Indonesia, Nigeria and South Africa rank first to fifth in terms of total number of incidence cases. Asia and Africa share the biggest burden of TB cases; 55% and 31%, respectively followed by small proportion in Eastern Mediterranean (6%), European region (5%) and America (3%).

In the year 2007, in addition to estimated 9.27 million new TB cases (first episode), there were additional 1.16 million subsequent episodes of TB (episodes occurring in patients who had already experienced at least one previous episode of TB in the past and who had received at least one month of anti-TB treatment). Among the total 10.4 million episodes of TB (first and subsequent), an estimated 4.9% or 511, 000 were cases of MDR-TB. Of these, 289, 000 were among new cases (3.1% of all new cases) and 221, 000 were among cases that had been previously treated for TB (19% of all previously treated cases). Of the total 511, 000 incident cases of MDR-TB in 2007, 349 000 (68%) were smear-positive cases, which are more likely to transmit the disease to others (WHO, 2009a). Mortality due to TB in HIV negative people were estimated to be 1.32 million (19.7 per 100, 000 population) in 2007, and there were an additional 456, 000 TB deaths among HIV-positive people the same year (WHO, 2009a).

According to WHO global 2009 TB report, Ethiopia had 314,267 estimated TB incidence in 2007, which ranked the country 7th from the top 22 highly TB burdened countries (**Table-1**, WHO, 2009a). Estimated TB incidence was 378 cases per 100,000 population and among them 163 cases per 100,000 populations were new smear positive cases. Ethiopian National Tuberculosis and Leprosy Control Program began to implement DOTS program in two zones in 1991 (USAID, TB profile 2009). In 2007, the national DOTS coverage reached 95% but case detection rate remains low (28%) when compared to 70% goal set by WHO (WHO, 2009a). This might be due to the limited diagnostic

capacity for TB. Meanwhile the treatment success rate reached 84% which is almost the 85% target set by WHO.

Table-1 Estimated epidemiological burden of TB, 2007

	POPULATION 1000s	INCIDENCE*				PREVALENCE*				MORTALITY				HIV PREV. IN INCIDENT TB CASES ^b %
		ALL FORMS		SMEAR-POSITIVE		ALL FORMS		HIV-NEGATIVE		HIV-POSITIVE				
		NUMBER 1000s	PER 100 000 POP PER YEAR	NUMBER 1000s	PER 100 000 POP PER YEAR	NUMBER 1000s	PER 100 000 POP PER YEAR	NUMBER 1000s	PER 100 000 POP PER YEAR	NUMBER 1000s	PER 100 000 POP PER YEAR			
1 India	1 169 016	1 962	168	873	75	3 305	283	302	26	30	2.5	5.3		
2 China	1 328 630	1 306	98	585	44	2 582	194	194	15	6.8	0.5	1.9		
3 Indonesia	231 627	528	228	236	102	566	244	86	37	5.4	2.4	3.0		
4 Nigeria	148 093	460	311	195	131	772	521	79	53	59	40	27		
5 South Africa	48 577	461	948	174	358	336	692	18	38	94	193	73		
6 Bangladesh	158 665	353	223	159	100	614	387	70	44	0.4	0.3	0.3		
7 Ethiopia	83 099	314	378	135	163	481	579	53	64	23	28	19		
8 Pakistan	163 902	297	181	133	81	365	223	46	28	1.4	0.9	2.1		
9 Philippines	87 960	255	290	115	130	440	500	36	41	0.3	0.3	0.3		
10 DR Congo	62 636	245	392	109	174	417	666	45	72	6.0	10	5.9		
11 Russian Federation	142 499	157	110	68	48	164	115	20	14	5.1	3.6	16		
12 Viet Nam	87 375	150	171	66	76	192	220	18	20	3.1	3.5	8.1		
13 Kenya	37 538	132	353	53	142	120	319	10	26	15	39	48		
14 Brazil	191 791	92	48	49	26	114	60	5.9	3.1	2.5	1.3	14		
15 UR Tanzania	40 454	120	297	49	120	136	337	12	29	20	49	47		
16 Uganda	30 884	102	330	42	136	132	426	13	41	16	52	39		
17 Zimbabwe	13 349	104	782	40	298	95	714	6.9	52	28	213	69		
18 Thailand	63 884	91	142	39	62	123	192	10	15	3.9	6.0	17		
19 Mozambique	21 397	92	431	37	174	108	504	10	45	17	82	47		
20 Myanmar	48 798	83	171	37	75	79	162	5.4	11	0.9	1.9	11		
21 Cambodia	14 444	72	495	32	219	96	664	11	77	1.8	13	7.8		
22 Afghanistan	27 145	46	168	21	76	65	238	8.2	30	0.0	0	0		
High-burden countries	4 201 761	7 423	177	3 245	77	11 301	269	1 058	25	339	8.1	14		

Adopted from WHO TB report 2009

1.2 Burden of TB/HIV

Since the emergence of HIV/AIDS pandemic; the prevalence, incidence, morbidity and mortality related to TB has been increasing. TB is the leading cause of morbidity and mortality among people living with HIV in Africa, and a major cause of death elsewhere. At least one-third out of 33.2 million people living with HIV worldwide is believed to be infected with *M.TB* and they are 20-30 times more at risk to develop active TB than those without HIV (WHO, 2009b).

Among the 9.27 million incident cases of TB reported in 2007, an estimated 1.37 million (14.8%) were HIV-positive (WHO, 2009b). In the same year, as in previous years, the African Region accounted for 79% of HIV-positive TB cases (South Africa accounted for

31% of cases in the African Region), followed by the South-East Asia Region (mainly India) with 11% of total cases. Mortality from TB among HIV-positive people account for 26 % (456,000) of total deaths due to TB that occurred in 2007.

It is well understood that early diagnosis and treatment of TB in People living with HIV has permanent importance. However, diagnosis of TB in HIV-infected patients is often difficult due to several reasons, such as frequently negative sputum smears, atypical radiographic findings, higher prevalence of extra pulmonary TB and resemblance to other opportunistic pulmonary infections (Sharma *et al.*, 2005). Sputum smear microscopy, which is fast, cheap and the most widely available method in the highly TB/HIV burdened developing countries, gives higher percentage of false negative results (Harries *et al.*, 1998).

1.3 Justification of the study

Diagnosing a TB patient is an entry point for TB control program. Laboratory diagnosis could be based on direct microscopy, culture (solid or liquid) and/or molecular detection of the bacteria with their own merits and demerits. Despite being the corner stone for TB diagnosis, smear microscopy lack sensitivity and specificity. On the other hand, culture systems have high sensitivity and specificity even though solid culture has lengthy incubation time (up to 8 weeks) and intensive media preparation procedure; and liquid culture is expensive and needs equipment maintenance.

Blood agar media, routinely used in microbiology laboratory, is cheap, with readily available ingredients and simple to prepare in any microbiology media preparation facility might be an alternative for TB diagnosis. Few studies conducted in recent years reported good sensitivity and specificity of blood agar media for primary isolation of *Mycobacterium tuberculosis* complex (Drancourt *et al.*, 2003; Drancourt and Raoult, 2007; Mathur *et al.*, 2009). Furthermore, other previous studies reported excellent performance of blood agar media for the drug susceptibility testing of anti-mycobacterium drugs. Thus this study was aimed to assess the suitability of blood agar media for TB diagnosis.

2. LITERATURE REVIEW

2.1 The bacteria

Mycobacterium species are non-motile, non-sporulated bacteria with high guanine plus cytosine (61-71%) containing DNA and high lipid containing cell wall. Some of the distinctive characteristics of mycobacteria like acid fastness (resistance to acid decolorization), resistance to injury (even for many antibiotics) and extreme hydrophobicity are strongly associated to the waxy coat of the bacteria. These, probably, contributes to the low growth of the bacteria by hindering nutrient intake (Barrera, 2007).

Due to the nature of the waxy cell wall, mycobacteria stain well in gram stain. Thus, special staining techniques like Ziehl-Neelsen staining, which uses acid decolorizing agents, are widely used to stain mycobacteria since the bacteria are resistance to acid treatment. Under light microscope, in smear stained with carbon fuchsin; mycobacteria appear red straight or slightly curved rods. Depending on growth condition and age of culture, mycobacteria may vary in shape from coccobacilli to long rods. Under microscopic observation, it is not possible to differentiate *Mycobacterium* at species level. However, rough colony growth on solid media, unlike smooth colonies of most non virulent mycobacteria and serpentine/cording feature of colonies under light microscope gives presumptive distinction of *M.TB* from other mycobacteria (Kent & Kubica, 1985; CLSI, 2008).

Tuberculin bacillus can build all its components from basic carbon and nitrogen sources (it is prototrophic) and it can also use already synthesized compounds as carbon and energy source (it is hetrotrophic) (Barrera, 2007). A study has shown that during the course of infection in mice, *M.TB* metabolism may shift from an aerobic, carbohydrate-metabolizing mode, to one that is more microaerophilic and utilizing lipids. The various physiological capabilities of the bacteria allow it to adapt and survive in harsh environmental condition.

In vitro, tuberculin bacillus can grow in a medium, which contains salt solution, glycerol or pyruvate (as a carbon source), ammonium ion and asparagines (as nitrogen source),

and micronutrients (Kent & Kubica, 1985). Albumin, which is normally provided by adding eggs or bovine serum albumin to the culture media, promotes the growth of these microorganisms. *In vitro*, *M.TB* is non fastidious. In fact, Robert Koch, in his famous research in discovering mycobacteria for the first time, used sterilized blood serum medium to cultivate the bacteria (Koch, 1882). In nature, mycobacteria grow successfully in high oxygen tension environment (in tissues like lung) whereas *in vitro* the organism favors 5-10% carbon dioxide environment. Unlike most bacteria, which divide every 30-60 minutes; *M.TB* divides in a very slow rate, which is once within 18-24 hours (WHO, 1998b). This explains the sub-acute to chronic evolution of the disease and the longer time requirement to get visible colonies *in vitro* culture (Barrera, 2007). Some of the reasons for slow growth of mycobacteria might be cell wall impermeability, which hinders nutrient uptake, low (10 fold lower than *E. coli*) RNA elongation rate (Harshey and Ramakrishnan, 1977) and retarded protein synthesis (Verma *et al.*, 1999). *M.TB* grows best at 37°C in neutral pH.

2.2 *Mycobacterium tuberculosis* complex (MTBC)

Numerous *Mycobacterium* species exist in the world, amongst most of them live and replicate in natural ecosystem and rarely cause disease. Only few of the species cause disease in higher vertebrates. *Mycobacterium leprae*, *M. lepraemurium*, *M. avium* subsp. *paratuberculosis* and members of MTBC are host-dependent mycobacteria that cannot replicate outside of their hosts (Barrera, 2007).

The members MTBC are mostly associated in causing TB in human. The complex includes *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. microti*, *M. canettii*, *M. caprae* and *M. pinnipedii* (Cataldi & Romano, 2007). Within the MTBC different etiologic agents have different host, zoonotic potentials and reservoirs. *M.TB*, *M. africanum* and *M. canettii* are primary pathogens of human; whereas *M. bovis* and *M. microti* mainly cause TB in animals and also cause disease in human. On the other hand, *M. caprae* and *M. pinnipedii* are pathogens of goats and seals, respectively. However, they have also been isolated from human (Thompson *et al.*, 1993; Kubica *et al.*, 2003).

2.3 Transmission and Pathogenesis

M.TB is carried in airborne particles called droplet nuclei generated when a person who has pulmonary and laryngeal TB disease coughs, sneezes, shouts, or sings (American Thoracic Society, 1990). The particles are approximately 1-5 μ m; normal air currents can keep them airborne for prolonged periods and spread them throughout a room or building (Wells, 1955). *M.TB* is usually transmitted through air, not by surface contact. Infection occurs when a susceptible person inhales droplet nuclei containing *M.TB*, and the droplet nuclei cross the mouth or nasal passages, upper respiratory tract, and bronchi to reach the alveoli (Jensen *et al.*, 2005).

During the inhalation of *Mycobacterium*, the body could either eliminate the bacteria using host immune response, or fail to eliminate the pathogen and develop active TB after some time, or manage to restrain the bacterial inside infected cell and lead to latency (Flynn & Chan, 2001). In the battle between the bacteria and host immune system, numerous immune components are involved. Macrophages, dendritic cells (innate immunity), CD4+ and CD8+ cells (cellular immunity) and IFN- γ , IL-12, TNF- α (cytokines) are among the major immune components involved in host immune response against *M.TB*. Thus, even though one third of the world population is infected with *M.TB* (Sudre *et al.*, 1992), only 5-10% develops active TB throughout their life time (Parrish *et al.*, 1998). The vast majority of TB infection goes to latency due to effective mechanism of host immune system.

In recent years, the emergence of HIV has fueled the incidence of TB and altered the course of infection (Sharma *et al.*, 2005). In early HIV infection, the course of TB infection in HIV positive patients is similar to immunocompetent HIV negative individuals. However, deterioration of immunity as the HIV disease progresses (with low CD4+ cell count) results in greater incidence of TB in HIV positive population. In addition, HIV positive patients are more likely to develop extra-pulmonary and disseminated TB due to the failure of host immune system to constrain *M.TB* in the granuloma (Lawn, 2005).

2.4 Tuberculosis Diagnosis

Diagnosing TB involves both clinical and laboratory procedures. Diagnosis using sign and symptoms are the first entry points for further investigation of the cause of disease. The most frequent signs and symptoms of TB are lack of appetite, low-grade evening fevers, cough and night sweats (MoH, 2008). Regarding respiratory signs and symptoms, the patient may complain of cough at any hour of the day, which is initially dry and later becoming productive with purulent or mucous expectoration and in some cases bloody sputum occur originating from lesions invading blood vessels. Chest pain can be localized and dependent on breathing movements (Hopewell, 2006). Presumptive diagnosis with sign and symptom needs to be confirmed by laboratory based diagnosis.

2.4.1 Smear Microscopy

Smear microscopy has remained as the corner stone for TB diagnosis since it first introduced by Robert Koch in 1882. There are a number of available mycobacterial staining techniques. However, staining based on the acid fast nature of the bacteria are the most widely used. They are based on the acid-alcohol decolorization resistant nature of the mycobacterial cell wall. Carbofuchsin and florochrome staining procedures are some examples of Acid Fast Bacilli (AFB) staining techniques. AFB smear microscopy is the most widely used method to detect active pulmonary TB specially in resource limited countries due to its availability, inexpensive cost and less time requirement for testing.

Ziehl-Neelsen staining

One of the famous Carbofuchsin AFB staining methods is Ziehl-Neelsen staining technique. In this technique, sputum smear prepared on slide will be treated by Carbofuchsin and heated. This will result in waxing the cell wall of the bacteria and allowing the stain to penetrate the cell wall. When treated with acid-alcohol, after 5 minutes of incubation without any heat, the primary stain will be retained only by mycobacteria (acid fast bacilli) and the rest will be decolorized. Counter staining with Methylene blue will stain the background blue and leaves the bacillus the only red stained bacteria in the slide. Finally, the slide will be observed under light microscope using 1000 magnification and the result is reported as recommended by WHO (1998a).

Despite being the most widely used diagnostic method in peripheral laboratories of developing countries, which are highly burdened by TB epidemic, Ziehl-Neelsen method misses two third of active TB cases (Waard & Robledo, 2007). The sensitivity of this method is low, and for a test to be positive, it requires 5,000-10,000 bacilli/ml of sputum (David, 1976). To overcome the possibility of reporting false negatives, the laboratory personnel are expected to read 300 fields per slide before reporting a result (Smithwick, 1976). However, this will increase work load and cause fatigue on the laboratorian. Sputum concentration is an alternative method to increase sensitivity of sputum smear examination. A significant improvement, in the proportion of positive AFB smear results, ranging from 7 % to 253 % has been reported due to application of concentration method (Angeby *et al.*, 2004).

Fluorochrome staining

The other AFB based staining for TB is fluorochrome staining. In the fluorochrome procedure, the prepared and heat fixed smear will be primarily stained with auramine-O, followed by acid-alcohol decolorization and finally counter stained using potassium permanganate. The AFB fluoresces yellow against a counter stain of potassium permanganate when observed under fluorescence microscope and it will allow easy identification of the bacilli (Kent & Kubica, 1985). Microscopic observation is done 250x or 450x magnification unlike 1000x used in direct microscopy. The lower magnification used in this technique allows to view larger field, helps to minimize the need to observe many fields and reduces reader fatigue. Fluorescent staining is more sensitive than Ziehl-Neelsen staining (Steingart *et al.*, 2006). The major disadvantage of this method is that the fluorescence fades with time, thus the slides must be read within 24 hours.

2.4.2 Mycobacterial Culture

Acid fast microscopy is based on the detection of any AFB, which could be *M.TB* or any other *Mycobacterium* species. On the other hand, culture is a technique based on the cultivation of live bacilli on appropriate culture media (WHO, 1998b). Robert Koch was the first person to invent a new solid media made up of potato and agar to cultivate *M.TB* (Koch, 1882). Depending on the decontamination method and the type of culture medium used, as few as ten viable tubercle bacilli might be enough to isolate *M.TB* using culture techniques (WHO, 1998b). This allows earlier detection of TB cases before they become infectious. However, culturing of *M.TB* may take up to 8 weeks due to very slow cell division of *Mycobacterium* (it divides every 18-24 hours) unlike other bacteria, which typically divide every 30-60 minutes.

Bacteriological culture not only solves the low sensitivity and specificity of smear microscopy but also allows performing drug susceptibility testing (DST) that assists in patient management. However, due to its expensive cost and the need for well established laboratory requirements, the use of culture technique for TB diagnosis should be selective (WHO, 1998b). WHO prioritized and recommended culture to be used as follows:

1. Surveillance of TB drug resistance as an integral part of the evaluation of control programme performance;
2. Diagnosis of cases with clinical and radiological signs of pulmonary TB where smears are repeatedly negative;
3. Diagnosis of extra-pulmonary and childhood TB;
4. Follow-up of TB cases who fail a standardized course of treatment and may be at risk of harboring drug resistant organisms; and
5. Investigation of high-risk individuals who are symptomatic, eg. Laboratory workers, health care workers looking after multidrug resistant patients.

Solid culture

The ideal medium for isolation of tubercle bacilli should be economical, simple to prepare from readily available ingredients, inhibit the growth of contaminants, support luxuriant growth of small numbers of bacilli and permit preliminary differentiation of

isolates on the basis of colony morphology (WHO, 1998b). However, no single medium fulfills all requirements and some species may grow on one media but not on the other.

There are a number of culture media to cultivate *M.TB*. Solid media can be categorized into two groups: egg based and agar based. Egg based media are easier to prepare and less expensive than agar based media. Egg based media support the growth of most mycobacterial strains, have low contamination rate and can be stored in refrigerator for several weeks. However, they are implicated in lengthy incubation requirement and when contamination occurs; it usually distorts the entire media (WHO, 1998b). On the contrary, agar based media have less contamination due to their simple chemical formulation, easy to read since the media are usually transparent and if incubated at 10% CO₂ atmosphere, positive culture could be obtained faster than on solid media. Disadvantages of agar based media include difficult media preparation (needs great care not to contaminate), pron for drying at the time of storage or incubation, since it is on plate, exposure of the medium for daylight results in formation of formaldehyde, which inhibits mycobacterial growth (Kent & Kubica, 1985). Thus, it is advisable to use both egg based and agar based media in combination for primary isolation of *Mycobacterium* (Kent & Kubica, 1985). Using media of two different types ensures maximum recovery of most strains of *Mycobacterium*. Egg based media, Lowenstein-Jensen (LJ) and Ogawa, and agar based solid media, Meddilebrok 7H-10 & 7H-11, are the most commonly used solid media worldwide for the isolation of *M.TB* (CLSI, 2008).

M.TB grows very well in protein rich media, such as egg-enriched media containing glycerol and asparagine, and agar or liquid medium supplemented with serum or bovine albumin (Kent & Kubica, 1985). Specimens collected from sterile body sites such as aspirated body fluids, blood and bone marrow can be concentrated and inoculated directly on to the culture media. However, specimens from non sterile body sites such as respiratory tract, where bacteria other than TB present as normal flora, needs proper decontamination prior to culturing (CLSI, 2008). Otherwise, the slow growing mycobacteria will be overwhelmed by normal flora and hinder their growth (Kent & Kubica, 1985). On the other hand, over decontamination (either treating specimen with

high concentration of decontaminant and/or prolonged treatment with routinely used concentration) may cause elimination of mycobacteria themselves. Thus, proper decontamination is vital in culturing *Mycobacterium*. Properly decontaminated sputum will be concentrated by spontaneous sedimentation or centrifugation and will be ready to be cultured on appropriate media.

Mycobacterial culture in LJ medium is one of the gold standard technique widely used for the isolation and DST of *M.TB* in the world (Kent & Kubica, 1985; WHO, 1998b; CLSI, 2008). LJ media can be prepared in every mycobacteriological laboratory can be and obtained commercially. A processed specimen inoculated on LJ media and monitored twice a week for the first 4 weeks then once a week until the end of 8 weeks for the growth of mycobacterial species. Since TB is a slow growing organism, positive result (colonies) usually appear after 3 weeks of incubation. On LJ media *M.TB* colonies appear as colorless, flat, dry and rough with irregular edges. Acid fastness can be confirmed by smear microscopy (CLSI, 2008).

Liquid culture

The use of liquid culture media for *Mycobacterium* cultivation was introduced in 1980 (CLSI, 2008). These techniques have high sensitivity and allow growth of *Mycobacterium* much faster than solid media. In a meta-analysis of 10 studies, the sensitivity and specificity of liquid culture system ranges from 81.5 - 85.5% and 99.6 - 99.9%, respectively (Cruciani *et al.*, 2004). Reports showed the time for culture positivity for all *M.TB* (both smear positives and negatives) ranged from 12.9-15.0 days in different liquid culture methods, which allow growth much faster than LJ solid media (27 days). In addition, liquid culture system allows the growth of some fastidious *Mycobacterium* like *M. genavense* (Siddiqui *et al.*, 1993). Furthermore, most of liquid culture media have long shelf life and they can be stored at room temperature.

Like all other laboratory methods, liquid culture system has some limitations. It is very expensive to install and maintain in most resource constrained countries, despite the high burden of the disease in these settings (WHO, 2007). The method is also associated with

high contamination rate (Chew *et al.*, 1998; Williams-Bouyer *et al.*, 2000; Chien *et al.*, 2000; Huang *et al.*, 2001; Oto *et al.*, 2008; Srisuwanvilai *et al.*, 2008). To make the liquid media more selective and minimize contamination, antimicrobials are usually added in the media such as PANTA (Polymixin B, Amphotericin B, Nalidixic acid, Trimetoprim and Azlocilic). PANTA inhibits most gram positive and gram negative bacteria and fungi (Siddiqi & Rusch-Gerdes, 2006). The other drawback of liquid culture was the use of toxic radioactive materials, as growth detection mechanism, and their disposal (Morgan *et al.*, 1983). Fortunately, now there are new liquid culture alternatives, which avoided the use of radioactive ingredients as a detection material. Instead they based their detection strategy on the release or consumption of natural components like carbon dioxide or oxygen (Tortoli *et al.*, 1999).

One of the examples of liquid culture media is BACTEC mycobacteria Growth Indicator Tube (MGIT) 960. It is rapid, sensitive and reliable method as of the previous liquid culture method (BACTEC 460). Unlike BACTEC 460, which uses radio active ingredients as growth detection mechanism, growth detection in MGIT is based on the consumption of oxygen in MGIT tube. Moreover, MGIT is a fully auto-machined technology, which allows continuous monitoring of growth. A number of studies have been conducted aiming to assess the performance of MGIT system for the isolation and DST of *M.TB* compared to the conventional solid culture media (Oto *et al.*, 2008; Srisuwanvilai *et al.*, 2008; Uddin *et al.*, 2009) and BACTEC 460 TB liquid culture (Ardito *et al.*, 2000). The system was reported to have comparable and even better results than the other methods (Tortoli *et al.*, 1999; Ardito *et al.*, 2000; Somoskovi *et al.*, 2000; Lu *et al.*, 2002; Srisuwanvilai *et al.*, 2008).

The principle of MGIT is based on measuring fluorescence due to consumption of oxygen, which was quenching fluorochrome (Siddiqi & Rusch-Gerdes, 2006). The MGIT tube contains sterilized 7.0ml of modified Middlebrook 7H9 broth base. In addition to the media, MGIT contains an oxygen-quenched fluorochrome (tris 4, 7-diphenyl-1, 10-phenanthroline ruthenium chloride pentahydrate) embedded in silicone at the bottom of the tube. PANTA and enrichment MGIT OADC (Oleic acid, Albumin, Dextrose and Catalase) or BACTEC

MGIT 960 Growth Supplement, is added to make the medium complete. The antimicrobial PANTA inhibits contaminants and the growth supplement is essential for growth of many mycobacteria, especially those belonging to MTBC. Once PANTA and growth supplement are added to the MGIT, inoculating processed specimens and incubate the tube at 37°C in BACTEC 960 instrument will be followed. During bacterial growth, free oxygen in the tube is consumed and the quenched fluorochrome is freed, which results in fluorescence when visualized under UV light. As a consequence in depletion of oxygen, any microorganism might give fluorescence. Thus, culture positivity should be confirmed by smear microscopy and/or culturing on blood agar media. The intensity of fluorescence is directly proportional to the extent of oxygen depletion. In case of *M.TB*, at the time of culture positivity, there are approximately $10^5 - 10^6$ colony forming units (CFU) per ml of medium. The instrument declares a tube negative if it remains negative for six weeks (Siddiqi & Rusch-Gerdes, 2006).

DST can be performed based on the same principle. Two MGIT tubes are inoculated with the test culture. A known concentration of a test drug is added to one of the MGIT tubes, and growth is compared with the MGIT tube without the drug (growth control). If the test drug is active against the isolated mycobacteria, it will inhibit the growth and thus there will be suppression of fluorescence, while the growth control will grow uninhibited and will have increasing fluorescence. Growth is monitored by the BACTEC 960 instrument, which automatically interprets results as susceptible or resistant (Siddiqi & Rusch-Gerdes, 2006).

In developed countries, liquid culture medium is standard of care for TB diagnosis and patient management (Anthony *et al.*, 2009). Despite its great value in improving patient management, the high cost associated in purchasing the machine, accessories and reagents makes it unaffordable for developing countries. Thus, WHO recommends countries to consider sustainable finance before buying such expensive equipment (WHO, 2007). Moreover, international guidelines recommend that all specimens inoculated on liquid culture media should also be inoculated on solid media too (WHO, 2007).

2.4.3 Identification Test

Most isolates, which cause disease in human, belongs to MTBC. Culture growth rate and colony morphology might allow presumptive characterization of MTBC from non-tuberculosis *Mycobacterium*. However, there is a need for definitive species characterization. Identification tests include phenotypic, genotypic and rapid identifications assays. Biochemical tests are the major component of phenotypic methods and they are the conventional methods to differentiate mycobacteria at species level. There is no single reliable biochemical test to distinguish MTBC from other mycobacteria. Therefore, a number of biochemical tests needs to be performed to characterize *Mycobacterium* at species level. Even though biochemical tests are cumbersome, still they are being used intensively in resource constraint areas.

On the other hand, genotypic methods are based on the amplification and sequencing of some targeted region of the *Mycobacterium*. Genotypic methods are more sensitive, fast and less labor intensive than conventional biochemical tests. However, like all other molecular assays they need special facilities to perform. Simple immunochromatography tests that can identify *M.TB* from liquid or solid culture by detecting species-specific secreted antigen, mycobacterial protein fraction from BCG 64 (MPB64) have recently been developed (CLSI, 2008). Only members of MTBC produce MPB64 protein.

One of the examples of rapid immunochromatographic assay for species identification is Capilla TB-Neo (TAUNS laboratories Inc, Numazu, Japan). The test is meant to differentiate MTBC from other mycobacteria (WHO, 2008b). Different studies reported Capilla had excellent performance in differentiating MTBC when compared with other established phenotypic or genotypic method (Wang, 2007; Ngamlert *et al.*, 2009). The principle of Capilla is based on antigen-antibody interaction. In a carrier strip colloidal gold labeled mouse anti-MPB64 monoclonal antibody is absorbed in specimen placing area, whereas mouse anti-MPB64 monoclonal antibody and anti-mouse immunoglobulin are immobilized on test and control area of the test device, respectively. When a specimen is dropped on the specimen pad the colloidal gold labeled anti-MPB64 monoclonal antibodies dissolve and bind to MPB64 antigens (if they are present in the

specimen) and form antigen-antibody complex (MPB64 antigen and anti-MPB64 antibody labeled with conjugate). The immune complexes continue to migrate along the strip and reach to testing and control area. Here, the antigen-antibody complex is trapped by immobilized anti MPB64 antibody and free conjugated MPB64 monoclonal antibodies trapped by anti-mouse immunoglobulin. Double red line on the test and control area is interpret as positive for MTBC and red line only on control area is interprets as negative, whereas no band in the test/control area is interpret as invalid and the test needs to be repeated.

2.4.4 Drug Susceptibility Testing (DST)

DST of *M.TB* needs to be done for MTBC isolates from all newly diagnosed patients (Tenover *et al.*, 1993; Styrk *et al.*, 1997), for patients at high risk of primary drug resistance and patients with another life threatening illnesses (American Thoracic Society, 1990). DST could also be requested if there is relapse, treatment failure and suspected drug resistance (Kent & Kubica, 1985). DST can be performed on processed clinical specimen (Direct Method) or from clinical isolates (Indirect method). DST could be either phenotypic or genotypic. Phenotypic method could be absolute concentration method, resistance ratio method or proportion method (Canetti *et al.*, 1969). All are based on measuring the level of resistance in solid or liquid culture.

Indirect proportion method in solid culture

Indirect proportion method is a gold standard and widely used DST method. It is based on measuring the drug resistance level of isolates in pure culture. The method allows the exact determination of resistant mutants present in the clinical isolates (Kent & Kubica., 1985). Several dilution of clinical isolates will be prepared and inoculated to control and drug containing media, incubated at 37°C and monitored for growth once a week (NCCLS, 2003). To determine the level of resistance, at least one of the dilutions must give countable colonies, 50-100 CFU. The counted colonies multiplied by the dilution factor will give the number of colonies for undiluted isolates. Then number of CFU in drug containing media divided by CFU in control media, considering the dilution factor, express the degree of resistance in percentage.

2.4.5 Other laboratory diagnostics

There are a number of new diagnostic methods for the detection of mycobacterial infection or disease. Among them, Line Probe Assay (LPA) from molecular assays and microscopic observation drug susceptibility (MODS) from non-commercialized will be discussed briefly.

Line Probe Assay (LPA)

Rapid identification of MTBC directly from clinical specimen tremendously shorten the time required to isolate *M.TB* in culture from weeks to few days. LPA is recommended by WHO for rapid screening of patients at risk of MDR-TB (WHO, 2008a). It is designed to detect MTBC and at the same time to detect RIF only or RIF and INH resistance directly from smear positive specimen or from culture isolates. LPA technology involves DNA extraction, gene amplification by PCR, probe-PCR product hybridization and colorimetric detection.

LPA shows more than 97% sensitivity and 99% specificity for the detection of RIF resistance alone or 90% sensitivity and 99% specificity in combination with INH (WHO, 2008a). LPA is one step detection and DST assay, fast (result within 1-2days) and relatively safe (may avoid growing live bacteria) method. However, it requires prior culturing of smear negative specimens. Additionally, there is a need for molecular diagnostic facility, for highly trained personnel and its high cost are some of the concerns about LPA (WHO, 2008a; WHO, 2010a).

Microscopic observation drug susceptibility (MODS)

The principle of MODS is based on growing *M.TB* in liquid culture and observing the typical cording characteristics of *M.TB* under microscope (Caviedes, 2000). MODS is designed for isolation of *M.TB* and DST of RIF and INH at the same time. It can be performed on smear positive specimens directly or indirectly from culture isolates. Both isolation and DST in MODS is achieved by inoculation of specimen to drug free and drug containing media simultaneously.

MODS showed high sensitivity and specificity (98 and 99%, respectively) for the detection of RIF resistance and less sensitivity (91%) for INH (WHO, 2010b). It has at same time detection and DST capacity; it is fast (result within 2 days-4 weeks) and cheap (non-commercial method). However, there is a need for conventional culturing of smear negative specimens. Moreover, MODS require additional staff skill, inverted microscope and consumables (WHO, 2010b).

Smear microscopy followed by solid culture (if it is available) are the most widely used diagnostic methods in developing countries. On the contrary, liquid culture and molecular assays have been utilized in developed countries for patient management. TB diagnostic methods other than those discussed above are available. All diagnostic methods have their strengths and weaknesses. Tables-2 summarizes currently available technologies for TB diagnosis, their advantages and disadvantages.

2.5 Tuberculosis Treatment

Treatment is required only for active TB; not latent TB, except for people living with HIV/AIDS who took Isoniazid (INH) prophylaxis (WHO, 2008c). Treating TB involves administration of combination drugs for months. Two biological features explain why combined drug therapy is more effective in curing TB than monotherapy. One feature is treatment of active TB with a single drug results in the selection of drug resistant bacilli and failure to eliminate the disease. The other feature is that different populations of tuberculin bacilli, each of which showing a distinct pattern of susceptibility for anti-tuberculosis drugs, may co-exist in a TB patient (Shamputa *et al.*, 2006). Moreover TB bacilli could present in the body of infected patients in two forms. Either they exist extracellularly in active dividing form and/or intracellularly in semi-active or dormant form, which shows the advantage to put a patient in combination therapy than monotherapy (Silva & Ainsa, 2007).

Table-2: Summary of the list of current methods available for TB diagnosis

Method	Advantages	Disadvantages
Clinical signs	Rapid diagnosis	Not specific, not conclusive, not always present
X-Ray	Readily available	Not specific or conclusive
Microscopy	Low cost, rapid diagnosis	Low sensitivity (up to 2/3 of pulmonary TB cases are false negative), difficult sample collection
Culture	Specific	Time consuming (up to 4-8 weeks), not always possible
PCR	Relatively quick, very specific	Relatively expensive, high level of training is required, expensive instrumentation, can detect latent disease
BACTEC	Specific, slow, 2-3 weeks	Expensive , not possible in all cases of TB
ELISA test kits	Quick(procedure time:110 min), reproducible, minimal training	Some equipment required
Rapid tests	Very quick (procedure time: only 15 min), minimal training , no special equipment required	Lower sensitivity compared to ELISA test kits, no quantitative results

Adopted from Tuberculosis book 2007

Drugs for treating TB are usually classified as first-line and second-line drugs. The main five first-line drugs are Isoniazid (INH), Rifampicin (RIF), Pyrazinamide (PZA), Ethambutol (EMB), and Streptomycin (SM) (WHO, 2010c). Second-line drugs include the Aminoglycosides Kanamycin and Amikacin, the Polypeptide Capreomycin, Para-aminosalicylic acid (PAS), Cycloserine, the Thioamides Ethionamide and Prothionamide and several Fluoroquinolones such as Moxifloxacin, Levofloxacin and Gatifloxacin (WHO, 2008d). The two core first line drugs, INH and RIF, act in individual population of bacilli. INH and RIF have high early bactericidal activity on actively dividing bacilli. In fact, INH was reported to kill 95% active bacilli within the first 2 days of treatment initiation (Mitchison, 2000). Appropriate dosage and combination of drugs are administered to TB diagnosed patients as per the WHO and country national guidelines. In Ethiopia, for the intensive phase of two months, INH, RIF, PZA and EMB; and for the continuation of six more months INH and EMB are used to treat TB (MOH, 2008).

3. OBJECTIVES

General objective

To assess the suitability of Blood Agar Medium for the diagnosis of TB.

Specific objectives

- To compare the performance of blood agar media versus LJ media and BACTEC MGIT 960 system for primary isolation of MTBC
- To compare the time required to grow MTBC in blood agar media versus LJ media and BACTEC MGIT 960 system
- To assess the performance of blood agar media for MTBC DST versus LJ media

4. MATERIALS AND METHODS

4.1 Study design

This was cross-sectional institution based test validation study. Specimens were collected consecutively from 108 patients referred to National TB Laboratory, Ethiopian Health and Nutrition Research Institute (EHNRI), for routine TB isolation and DST. Both culture and DST were done in National TB Laboratory, EHNRI. All specimens were inoculated on LJ, Blood agar media and MGIT in parallel. Those samples with positive TB culture results were tested for drug susceptibility of 1st line antimycobacterial drugs using LJ and Blood agar media.

4.2 Study population

The study participants were patients referred to the National TB Referral Laboratory, EHNRI, for TB culture and DST between February 2010 and May 2010. A total of 107 samples were collected from 107 patients. Due to the demanding nature (both technically and financially) of TB culture, we used convenient sampling strategy to get desired sample size and composition. Accordingly, 56 smear negative and 51 smears positive patients were included in the study consecutively. The sex composition of study participants was 56.7% male and 43.3% female. The age of participants ranged from 15 to 74 years with a mean age of 35.1 years. The study participants were composed of new, relapse, treatment failure, defaulters and multidrug resistance cases.

4.3 Specimen types

Clinical specimens were collected from both pulmonary and extra-pulmonary TB suspected patients. The vast majority of specimens collected was sputum (91.4%) followed by pus (4.8%), plural fluid (1.9%), bronchial lavage (0.95%) and lung aspirate (0.95%). Sputum and other pulmonary source specimens' account for 93.3% of the total specimen and only 6.7% of the samples were from non pulmonary sources.

4.4 Specimen processing

Specimens for this study were collected either from patients directly referred to EHNRI for TB culture, or specimens were collected from patients elsewhere and referred to EHNRI TB laboratory. Either ways, clinical specimens were collected from patients as recommended by WHO (WHO, 1998b). Samples collected elsewhere were transported to the laboratory, and accepted after assessing the quality of specimens (integrity of the specimen container, volume of specimen and proper labeling).

N-acetyl L-cysteine sodium hydroxide method (NALC-NaOH) was used to decontaminate clinical specimens (Kent & Kubica, 1985; WHO, 1998b). NALC-NaOH method allows decontaminating clinical specimens with low concentration of NaOH (1%), which in turn minimize the risk of killing bacilli in the process. NALC liquefies sputum sample and facilitates the decontaminating action of NaOH.

Equal volumes of clinical specimens and NALC-NaOH solution were mixed (5ml sputum and 5ml of 2% NALC-NAOH) in 50ml Falcon tube, briefly vortexed and incubated for 15 minutes at room temperature. At the end of incubation, phosphate buffer solution (PBS) was filled to the 50ml mark of Falcon tubes, mixed by inversion to neutralized NaOH and stop decontamination. Then the Falcon tubes were centrifuged for 15 minutes at 3000rpm in Allerga® X-15R refrigerated centrifuge (Beckman Coulter, Marca Reg, USA), which has a cooling system to avoid heat buildup due to centrifugation that is distractive for the bacilli (Kent & Kubica, 1985). Finally, the supernatants were decanted and the sediments were re-suspended in 1ml of PBS and made ready for inoculation. In the entire decontaminating process, two negative controls (tube without any specimen) were included as a process quality control.

4.5 Smear examination

Direct smear examination using Ziehl-Neelsen staining was used at different stages of the study. First, smears prepared directly from processed specimens were stained and examined for the presence of AFB to classify specimens into smear positive and smear

negative. Second, smears were prepared from culture suspension of all culture positive specimens to confirm acid fastness according to WHO, MoH guidelines (WHO, 1998a).

4.6 Media preparation

4.6.1 Plain Media

Plain media are those media (Blood agar, LJ and MGIT) without anti-tuberculosis drugs which were used for primary isolation of MTBC and also used as comparison media in DST.

4.6.1.1 Blood agar media

Blood agar base (Oxoid, Hampshire, England) was used to prepare 5% sheep blood agar media according to manufacturer instruction with some modifications.

1. 40g blood agar base was mixed in a liter of distilled water and autoclaved at 121°C for 15 minutes.
2. The media was allowed to cool to 45-50°C.
3. 50ml of freshly collected defibrinated sheep blood was added to the base
4. 60ml of PANTA antibiotic (Becton Dickinson and company, Sparks, MD, USA) was added to the media.

N.B. This step is not included in the routine blood culture media preparation procedure.

5. Quickly 10-12ml of blood agar media was dispensed to 30ml McCartney tube and allowed to form a slant.
6. All blood agar media were incubated at 37°C for 24 hours to check sterility.
7. Blood agar media without any growth after 24 hours incubation at 37°C were kept at 2-8°C until used.

4.6.1.2 Lowenstein-Jensen media

Lowenstein-Jensen media were prepared according to WHO guideline (WHO, 1998b). The contents of the mineral salt solution: Potassium dihydrogen phosphate anhydrous, Magnesium sulphate and Glycerol were from Sigma (Sigma chemical Co.

st.Louise, MO, USA), and Magnesium citrate and Asparagine were from BDH (BDH laboratory supplies, Poole, England).

4.6.1.3 BACTEC MGIT 960

Unlike the above two solid media, the MGIT tubes contain readymade 0.7ml of sterilized Middlebrook 7H9 broth base. An enrichment, MGIT OADC or MGIT Growth supplement and antibiotic PANTA were added to the MGIT media at the time of inoculation to make the media ready for use. All testing components were products of Becton Dickinson and Company, Sparks, MD, USA.

4.6.2 Drug media

4.6.2.1 Drug preparation

The four anti-tuberculosis drugs (Isoniazid, Rifampicin, Streptomycin and Ethambutol) were prepared as follows.

Isoniazid: 0.1g INH powder (Sigma, St. Louise, MO, USA) was dissolved in 10ml sterile water to prepare stock solution-I (Sol. I) then allocated to nunc tubes in 1ml volume and stored at -20°C until used. At the time of media preparation, stock Sol. I was then diluted to 1:10 (1.0 ml Sol. I was added to 9ml sterile water) to prepare solution-II (Sol. II). Sol. II further diluted to 1:10 by adding 1.0ml Sol. II to 9ml sterile water to prepare solution-III (Sol. III). Appropriate volume of Sol. III was added to the media to get 0.2µl final INH drug concentration in the media (WHO, 2009c).

Rifampicin: 0.02g RIF powder (Sigma, St. Louise, MO, USA) was dissolved in 50ml of Dimethylsulfoxid (DMSO)(Merck, Germany) to prepare stock solution-I (Sol. I). Sol.II was prepared by further diluting 2ml of Sol.I to 18ml of distilled water. Appropriate volume of Sol. II was added to the media to get 0.40µl final RIF drug concentration in the media (WHO, 2009c).

Streptomycin: 0.125g SM (Sigma, St. Louise, MO, USA) was dissolved in 10ml distilled water to prepare stock solution-I (Sol. I) then allocated to nunc tubes in 1ml volume and

stored at -20°C until used. At the time of media preparation, stock Sol. I was then diluted to 1:10 (1.0 ml Sol. I was added to 9ml sterile water) to prepare solution-II (Sol. II). Appropriate volume of Sol. II was added to the media to get 4.0µl final SM drug concentration in the media (WHO, 2009c)

Ethambutol: 0.1g EMB powder (Sigma, St. Louise, MO, USA) was dissolved in 100ml sterile water to prepare stock solution-I (Sol. I) then allocated to nunc tubes in 1ml volume and stored at -20°C until used. At the time of media preparation, stock Sol. I was then diluted to 1:10 (1.0 ml Sol. I was added to 9ml sterile water) to prepare solution-II (Sol. II). Appropriate volume of Sol. II was added to the media to get 2.0µl final EMB drug concentration in the media (WHO, 2009c)

4.6.2.2 Blood agar media

For DST, plain blood agar media and anti-TB drugs containing blood agar media were prepared as followed.

1. Control blood agar media were prepared exactly as that of plain blood agar media devoid of PANTA.
2. Anti-TB drugs (INH, RIF, EMB and SM) containing media were prepared as followed
 - i. 5% blood agar media was prepared by dissolving 40gm of blood agar base in 1000ml of distilled water in four flasks for the four drugs, autoclaved at 121°C for 15 minutes, and 50ml of fresh defibrinated sheep blood was added each of four flasks.
 - ii. INH, RIF, EMB and SM solution were added to their designated media at final concentration of 0.2µl, 40µl, 2.0µl and 4.0µl, respectively.
 - iii. Quickly 10-12ml of the blood agar media were dispensed on 30ml McCartney tubes and allowed to form a slant.
 - iv. The blood agar media were kept in refrigerator (2-8°C) until use.

4.6.2.3 Lowenstein-Jensen media

Drug containing LJ media were prepared in exactly the same way as that of plain LJ media except the addition of anti-tuberculosis drugs. LJ mineral salt solution and egg homogenous were prepared exactly as that of plain LJ media preparation. Then, before the media were dispensed to the McCartney tubes, individual anti-TB drugs (INH, RIF, EMB and SM) were added to their respective flasks at the final concentration of 0.2µl, 40µl, 2.0µl and 4.0µl, respectively. Finally, media were dispensed to 14ml McCartney tube, inspissated and stored at 2-8°C until used.

4.7 Isolation of *Mycobacterium tuberculosis*

One hundred and seven clinical specimens were cultured on each of Blood Agar, LJ and MGIT media for the isolation of MTBC. The process followed for culturing MTBC in each media will be discussed as follows.

4.7.1 Isolation on Blood agar media

One hundred microliter of each properly processed specimen was inoculated to blood agar media in duplicate. Start and end controls were included in each run as a quality control tool. Inoculated media were incubated at 37°C in Binder incubator (Binder, Tuttlingen, Germany) for up to 8 weeks and culture was inspected for visible colonies 3 times a week (weekdays). Culture positivity was confirmed for acid fastness using Ziehl-Neelsen staining and then stored at -20°C until further use.

4.7.2 Isolation on Lowenstein-Jensen media

The procedure followed here was exactly the same as that of blood agar media. One hundred micro liter of each properly processed specimen was inoculated to LJ media in duplicate. Start and end controls were included in each run as a quality control tool. Then, the inoculated media were incubated at 37°C in Binder incubator (Binder, Tuttlingen, Germany) for up to 8 weeks and culture was inspected for visible colonies 3 times a week (weekdays). Culture positivity were confirmed for acid fastness using Ziehl-Neelsen staining and then stored at -20°C until further use.

4.7.3 Isolation on BACTEC media

All processed specimens were inoculated to MGIT tubes in 500µl volume, placed in BACTEC MGIT 960 machine (Becton Dickinson and Company, Sparks, MD, USA) and incubated at 37°C for 42 days. Growth in MGIT tube was monitored continuously and culture positivity was flagged by the machine. BACTEC machine registers the date and time of culture positivity automatically. All culture positive isolates were cultured on blood agar media to rule out any contaminants and also examined to confirm the presence of acid fast bacilli by Ziehl-Neelsen staining.

4.8 Species identification test

Capilla TB Neo (TAUNS Laboratories Inc, Numazu, Japan), Rapid immunochromatographic device was used to determine clinical isolates to species level. Isolates either from MGIT liquid culture or from solid culture (blood agar or LJ media) were used to do the testing. One hundred microliter culture suspensions from MGIT tube were directly applied to the test device. For cultures grown on solid media, 1µl of bacterial colony from solid media was suspended in 0.2ml extraction buffer supplied by the manufacturer and 100µl of the culture suspension was dropped to the testing device. The result was interpreted after 15 minutes of incubation at room temperature. Development of two purple bands (one each in test and control area) on the testing device was interpreted as positive for MTBC; development of only one band in the control area was interpreted as negative for MTBC and no band on the test device was considered as invalid result and retested (**Figure-1**).

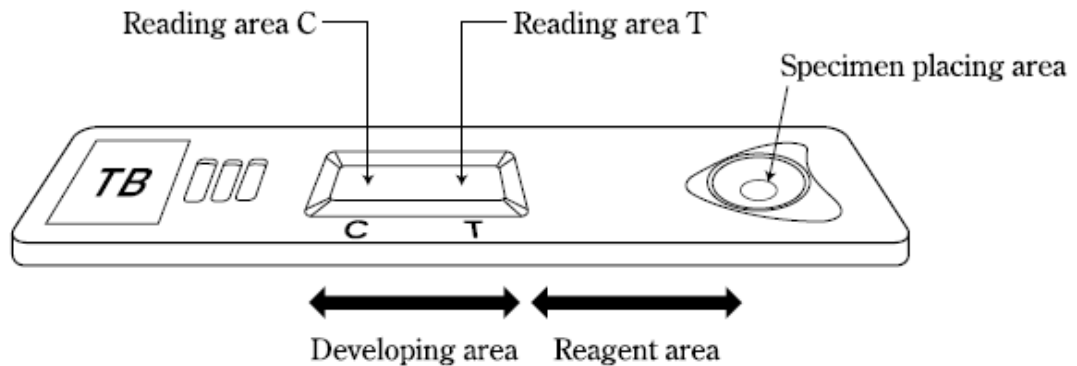


Figure- 1: Capilla immunochromatographic device for MTBC identification

4.9 Drug Susceptibility Testing

Blood agar media and Lowenstein-Jensen media

DST was done based on modified proportion method (NCCLS, 2003). One loop full colonies scraped all over the cultured strain were suspended in 2ml of sterile water in a sterilized screw capped tube containing 5-7 glass beads. Then, the suspension was vortexed for 10 seconds and left for 15-30 minutes to allow larger aggregates of bacteria to settle. Four milliliter of sterile water was added to the culture suspension and the supernatant was transferred to another tube with similar dimension as that of McFarland tube for comparative purposes. Bacterial suspensions were adjusted to match McFarland standard No.1 turbidity. From culture suspension matched to McFarland No.1, 100 fold dilution (10^{-2}) and 10,000 fold dilution (10^{-4}) were prepared. One hundred microliter of culture suspension from 10^{-4} and 100 μ l from 10^{-2} dilutions were inoculated separately to individual control media and 100 μ l culture suspensions from 10^{-2} dilution were inoculated to each 4 anti-tuberculosis drug containing media (INH, RIF, SM and EMB). Then, all inoculated media were incubated in Binder incubator (Binder, Tuttlingen, Germany) at 37°C up to 6 weeks and growth was monitored once a week. To calculate the percentage of resistant, the numbers of colonies on control media should fall between 50-150 colonies. The percentage of resistance strains was calculated by dividing the

number of colonies on drug media by the number of colonies on control media. The proportion of resistance bacteria more than or equal to 1% was taken as resistant and the proportion of bacteria less than 1% was considered as susceptible (Kent and Kubica, 1985; NCCLS, 2003).

4.10 Quality control

Irrespective of the type of media used, start and end control (negative controls) and H37Rv (known *M.TB* positive control) were included in each test run. Prior to interpreting test results, start and end control tubes were assessed for growth. Growth on start and end control indicates contamination in the laboratory procedure and the entire run considered as invalid and the tests need repetition. In contrast, known *M.TB* (strain H37Rv) was used to check the ability of the media to support proper growth of *M.TB* or not. If growth of known *M.TB* positive control is not observed, it indicates that the media do not support the growth of the bacteria, and then the entire run is considered invalid and needs repetition with fresh newly prepared culture media. Thus, before interpreting isolation results, validity of each test run was checked. H37Rv strain was also included in DST of each run. Since H37Rv is sensitive to all anti-tuberculosis drugs, its growth on control tubes but not on drug containing tubes was confirmed prior to interpreting DST results.

4.11 Statistical Analysis

Demographic data and patients' history obtained from laboratory request form, and laboratory results were captured by Microsoft office Excel 2007 and recording formats. Statistical analysis was performed using STATA (version 9.2) and Microsoft office Excel 2007. Student's t-test was used to detect statistically significant differences in time for culture positivity. On the other hand, Kappa-value was used to measure agreement between assays.

5. ETHICAL CONSIDERATION

The proposal was reviewed and approval was obtained from Biotechnology Program, Addis Ababa University and Scientific Ethical Review Office, Ethiopian Health and Nutrition Research Institute. Socio-demographic data were collected from patients' laboratory request forms and culturing on blood agar media were done together with the routine TB culturing process in the laboratory. No information and/or additional specimens were requested from the patients. Therefore, there was no harm for the study participants.

6. RESULT

This study is the first of its kind to see the performance of 5% sheep blood agar media for TB diagnosis in Ethiopia. It was a cross-sectional study conducted at National Tuberculosis Laboratory, EHNRI. The study covered both isolation of MTBC from clinical specimens and anti-tuberculosis DST for confirmed MTBC isolates. Blood agar media was compared with LJ solid media and MGIT automated liquid culture system for the isolation of *Mycobacterium* and only with LJ media for DST. Analysis was done on 105 samples excluding 2 samples because of persistent contamination after proper reprocessing.

6.1 Smear microscopy

Based on AFB smear microscopy results, the study participants were categorized into AFB smear positive and AFB smear negative. From the total 105 specimens collected 54 (51.4%) were AFB smear negative and 51 (48.6%) were AFB smear positive. The bacillary load for AFB smear positive specimens were 1+ for 14 (27.5%) of the specimens, 2+ for 13 (25.5%) of the specimens and 3+ for 24 (47%) of the specimens.

6.2 Performance of blood agar media for primary isolation of *Mycobacterium* species

Colonies of MTBC showed distinct characteristics of rough, tiny, dry, and colorless (whitish yellow) appearance when grown on blood agar media. The colonies were numerous in number but did not have luxurious growth.

The performance of 5% sheep blood agar media for the primary isolation of mycobacteria was assessed considering LJ media as a gold standard method. The sensitivity, specificity, positive predictive value and negative predictive value of blood agar media were 98, 98.2, 98 and 98.2%, respectively (**Table-3**).

On the other hand, the performance of 5% sheep blood agar media for the primary isolation of *M.TB* was assessed considering MGIT media as a gold standard method. The sensitivity, specificity, positive predictive value and negative predictive value of blood

agar media were 87.2, 100, 100 and 87.2%, respectively (**Table-3**). The sensitivity, specificity, positive and negative predictive value of LJ media was exactly the same (in 100% agreement) as that of blood agar media when compared to MGIT.

The recovery rate of each three methods (LJ, Blood agar and MGIT) was calculated by adding up all isolates recovered by any of the three methods after confirming culture positivity by AFB smear microscopy. From a total of 105 clinical specimens cultured 57 isolates were recovered by the three methods. However, two isolates were positive by the two solid media but invalid by MGIT because of persistent contamination even after proper re-processing. Thus, these two isolates were excluded from analysis and comparisons were done with the remaining 55 isolates. The recovery rate of MGIT was 100% followed by LJ and blood agar media with equivalent recovery rate, 90.9% (**Table-4**).

Table 3: Performance of blood agar media for the isolation of *Mycobacterium*

Blood agar media	Gold Standard Methods	
	LJ media % (95% CI)	MGIT % (95% CI)
Sensitivity	98 (88-99.9)	87.2 (74.9-94.3)
Specificity	98.2 (89-99.9)	100 (90.7-100)
Positive predictive value (PPV)	98 (88-99.9)	100 (90.7-100)
Negative predicative value (NPV)	98.2 (89-99.9)	87.2 (74.9-94.3)

N.B: CI= confidence interval

Table 4: Recovery rate of *Mycobacterium* species on blood agar, LJ and MGIT

	Both AFB + & -	AFB +	AFB -
	n (%)	n (%)	n (%)
Total isolates	55	47	8
Blood agar	50 (90.9)	46 (97.9)	4 (50)
LJ	50 (90.9)	45 (95.7)	5 (62.5)
MGIT	55 (100)	47 (100)	8 (100)
Both	47 (85.5)	44 (93.6)	3 (37.5)

N.B: n= number of isolates

%= isolates in individual media divided by total isolates times 100.

6.3 Time to detection (TTD) of mycobacterial isolates

Time for culture positivity on blood agar media ranged from 7 to 32 days with mean time to detection 17.3 days (15.8-18.7 days 95% CI). On the other hand, culture positivity in LJ media ranged from 10-41 days with mean time to detection 22.7 days (20.8-24.6 days 95% CI) and MGIT ranged from 4-28 days with mean time for detection 9.3 days (7.9-10.6 days 95% CI). The variations of TTD between each diagnostic test were statistically significant (P-value < 0.001). The fastest of all was MGIT followed by Blood agar media then LJ media (**Table-5 & 6**).

Table 5: Time to culture positivity

	Mean time to detection in days(range)		
	Both AFB + & -	AFB+	AFB-
Blood agar	17.3 (7-32)	17.2 (7-32)	18.5 (12-25)
LJ	22.7 (10-41)	22.3(10-41)	26.4 (20-38)
MGIT	9.3 (4-28)	8.1(4-19)	15.6 (5-28)

Note: time to culture positivity in solid media is the time when visible colonies 1st appear on the media; but on MGIT, it is the time machine flagged culture as positive.

In MGIT media, 46% and 90% of the isolates were recovered within the first and second weeks of incubation, respectively. On the other hand, on blood agar media only 4% and 28% recovery was achieved in the first and second weeks of incubation, respectively, whereas none isolates were recovered in the first week of incubation on LJ media and only 10% was recovered in the second week of incubation. LJ media required one more week to reach the 82% recovery rate that was achieved by blood agar media in 3rd week.

Table 6: Recovery rate of *Mycobacterium* species/ weeks of incubation

Incubation time in weeks	MGIT % recovered	Blood agar media % recovered	LJ media % recovered
1 st	46	4	0
2 nd	90	28	10
3 rd	98	82	58
4 th	100	98	82
5 th	-	100	96
6 th	-	-	100

N.B : % Recovery/weeks = isolates recovered in week/weeks by individual assay divided by total isolates recovered by the same assay

6.4 Species identification

In this study species identification was done using Capilla TB-Neo, rapid immunochromatographic assay, which tremendously improves diagnosis by shortening the time required for species identification that avoid work load related to tedious series of biochemical tests. A total of 57 samples, which were culture positive with any of the three culturing methods, were tested by Capilla to differentiate MTBC from NTM. Out of the total 57 culture positive samples 55 (96.5%) were MTBC and the rest 2 (3.5%) were NTM.

6.5 Performance of blood agar media for DST

DST was done for 43 blood agar, LJ culture and Capilla positive isolates. The main first line anti-tuberculosis drugs (INH, RIF, SM and EMB) were included in this study. The performance of blood agar media for *M.TB* DST was good when compared with the gold standard LJ media. The sensitivity, specificity, positive and negative predictive value of the basic anti-tuberculosis drugs was 100, 88.9, 97.1 and 100%, respectively for INH whereas 100% for all parameters for RIF. The sensitivity, specificity, positive and negative predictive value for SM and EMB were 89.2, 100, 100, 60 and 96.8, 100, 100 and 92.3%, respectively (**Table-7**).

Agreement between blood agar media and LJ media were found to be 97.7% (95% CI 86.2-99.9%) for INH, 100% (95% CI 89.8-100%) for RIF, 90.7% (95% CI 77-97%) for SM and 97.7% (95% CI 86.2-99.9%) for EMB. The overall agreement for all the 4 drugs between blood agar and LJ media, was 96.5% (95% CI 92.2-98.6%). Table-8 shows details of agreements for each drug with corresponding kappa-values.

Table 7: Performance of blood agar media for DST of MTBC

	INH	RIF	SM	EMB
	% (95% CI)	% (95% CI)	% (95% CI)	% (95% CI)
Sensitivity	100(87.4-100)	100(86.3-100)	89.2(73.6-96.5)	96.8 (81.5-99.8)
Specificity	88.9(50.7-99.4)	100(69.9-100)	100(51.7-100)	100(69.9-100)
PPV	97.1(83.4-99.6)	100(86.3-100)	100(87-100)	100(85.9-100)
NPV	100(59.8-100)	100(69.9-100)	60(27.4-86.3)	92.3(62.1-99.6)

N.B: CI= confidence interval

Table 8: DST concordance between assays

Drugs	Blood agar media	LJ media		Agreement % (kappa)
		Resistant	Susceptible	
INH	Resistant	34	1	97.7 (0.927)
	Susceptible	0	8	
RIF	Resistant	31	0	100 (1.000)
	Susceptible	0	12	
SM	Resistant	33	0	90.7 (0.697)
	Susceptible	4	6	
EMB	Resistant	30	0	97.7 (0.944)
	Susceptible	1	12	

N.B: % Agreement= no. concordance by blood agar and LJ media divided by the total concordance/discordance times 100

6.6 Contamination rate

Contamination rate was calculated by dividing fully contaminated tubes by total tubes inoculated times 100. The contamination rate in blood agar media was the lowest 5.1% (11/216) followed by LJ media 9.7% (21/216) and MGIT 14.8% (16/108).

7. DISCUSSION

In the current study the vast majority of clinical specimens were sputum and other specimens from pulmonary sources (93.3%). The high frequency of sputum and other pulmonary source specimens might be explained by the overwhelming incidence of pulmonary TB over extra-pulmonary TB. In the selection of AFB positives, the representation of different bacillary load was balance by including samples with 1+, 2+ and 3+ AFB results. This was aimed to void the bias associated to overestimate sensitivity by only using high bacillary load specimens.

The performance of blood agar media was compared with two gold standard methods with different principle. Based on the evaluation, blood agar medium has excellent performance for primary isolation of *M.TB* compared to the gold standard LJ solid media with a sensitivity of 98% as reported elsewhere with 98.9% sensitivity (Drancourt & Raoult, 2007) and better than 94.2% sensitivity by Mathur et al's (2009). Blood agar media has also shown specificity, PPV and NPV of 98.2, 98 and 98.2%, respectively. From the total of 105 cultured specimens, blood agar media gave only 2 discordant results. One of the samples, which was AFB negative, was also negative with blood agar media but positive with LJ media. The other one, which was AFB positive, was also positive by blood agar media but negative with LJ media. Both of the discordant samples were positive by MGIT. Thus, in actual terms, blood agar media missed only one positive sample. Blood agar media had overall agreement of 98.1% for primary isolation of *Mycobacterium* species with LJ medium, 97.2% on AFB positive and 98% on AFB negative specimens.

The second performance comparison for primary isolation of *Mycobacterium* species was done between blood agar media and BACTEC MGIT 960 liquid culture system. Assessing blood agar media performance with improved automated culture system will give better performance insight about the method. In numerous previous studies, better sensitivity of liquid culture systems over solid culture system was reported (Tortoli *et al.*, 1999; Ardito *et al.*, 2000; Somoskovi *et al.*, 2000; Lu *et al.*, 2002; Srisuwanvilai *et al.*, 2008). Similar results were obtained in the present study. The sensitivity, specificity, PPV

and NPV of blood agar media was 87.2, 100, 100 and 87.2% when compared to MGIT. Interestingly the performance of LJ media in comparison to MGIT was also exactly the same as that of blood agar media. In comparison to MGIT, the sensitivity of 87.2% for both blood agar and LJ media found in the current study was inline with 87.1% sensitivity of LJ media when compared to MGIT by Lee et al., (2003). The superiority of MGIT might be due to the machine culture detection principle the method used and the highly enriched media that liquid culture systems use. Lee et al also reported that Middlebrook 7H11 solid media had 75.7% sensitivity compared to MGIT, while LJ had 87.1%. Based on the similar performance of blood agar and LJ media in this study, it is possible to say blood agar media is superior than the conventional Middlebrook 7H10 solid media for isolation of *M.TB*.

The ability of each individual assay to recover mycobacterial species was calculated by adding up all isolates recovered by respective media. Based on this, MGIT recovered all 55 (100%) of the isolates, while both blood agar and LJ media recovered 50 (90.9%) of the isolates. In other studies the recovery rate of MGIT ranged from 80 to 100% (Tortoli *et al.*, 1999; Ardito *et al.*, 2000; Lee *et al.*, 2003; Balabanova *et al.*, 2009). On the other hand, the recovery rate of LJ was reported to range from 38 to 82% when compared to MGIT (Tortoli *et al.*, 1999, Ardito *et al.*, 2000; Somoskovi *et al.*, 2000; Balabanova *et al.*, 2009). In the current study, the recovery rate of LJ media was found to be better than the finding of those reports. There was no previous study, which compared blood agar with MGIT broth automated system, but one study compared blood agar media with BACTEC 9000 MB broth automated system and reported superior performance (98.9 versus 92.6%) of blood agar media over BACTEC 9000 MB system (Drancourt & Raoult, 2007).

The recovery rate in MGIT, blood agar and LJ media which is 100, 97.9 and 95.7%, respectively was excellent for AFB positive samples but in AFB negative samples, MGIT maintained 100% (8/8) recovery rate, whereas LJ and blood agar media declined to 62.5% (5/8) and 50%(4/8) recovery rates, respectively. High recovery rate of MGIT on AFB negative *M.TB* was also reported to be 97% (Srisuwanvilai *et al.*, 2008), 95.1%

(Somoskovi *et al.*, 2000) and 91.1% (Uddin *et al.*, 2009). On the other hand, lowered recovery rate by LJ media for same specimens were reported as 59% (Srisuwanvilai *et al.*, 2008), 71.1% (Uddin *et al.*, 2009) and 75.6% (Somoskovi *et al.*, 2000), which is in line with our findings.

There were 6 AFB positive but culture negative samples by either of the methods. Out of the 6 culture missed samples, 3 were missed by all the three methods, 2 by the two solid media and 1 by LJ media only. Most of these samples had low bacillary load (5 out of 6 were AFB grade 1+) and all of them were from old TB cases. The possible explanation of this phenomenon could be the high probability of these patients being on therapy and this might have resulted in low bacillary load plus growth inhibition, which leads to culture negativity.

The time to detection (TTD) of mycobacterial isolates was assessed for blood agar, LJ media and MGIT. Accordingly, TTD on blood agar media ranged from 7-32 days unlike 10-41 days on LJ media. Mean TTD on blood agar was 17.3 ± 1.4 days, which is faster than 22.7 ± 1.9 days on LJ media. This finding was in support of Mathur *et al.*'s mean TTD of 13.6 ± 5.2 days on blood agar and 20.4 ± 5.1 days on LJ media (Mathur *et al.*, 2009). Drancourt *et al.* also reported recovery of M.TB isolates within 2 weeks of incubation on blood agar media (Drancourt *et al.*, 2003). Another study by Drancourt and Raoult reported slightly higher median TTD 19 ± 5 days on blood agar but still much faster than BACTEC 9000 MB broth media, 26 ± 6 days (Drancourt & Raoult, 2007).

Liquid culture media was reported to isolate *Mycobacterium* species much faster than conventional solid media (Tortoli *et al.*, 1999; Ardito *et al.*, 2000; Somoskovi *et al.*, 2000; Srisuwanvilai *et al.*, 2008; Rodrigues *et al.*, 2009). In this study, it only took 4-28 days with mean TTD of 9.3 days for MGIT to give positive TB culture. In agreement with the present study findings, in previous studies MGIT was reported to detect *Mycobacterium* species with mean TTD of 9-14 days (Tortoli *et al.*, 1999; Somoskovi *et al.*, 2000; Ardito *et al.*, 2000; Srisuwanvilai *et al.*, 2008; Balabanova *et al.*, 2009; Rodrigues *et al.*, 2009). MGIT decreased time required to isolate *Mycobacterium* species

to about one week as compared to blood agar media; and about two weeks as compared to LJ media.

The vast majority of the isolates were recovered within 2 weeks by MGIT (90%), 3 weeks by blood agar (82%) and 4 weeks by LJ media (82%). Our finding with MGIT 90% recovery rate within 2 weeks was in lined with recent report of Rodrigues et al (2009) who found 91% recovery rate in 2 weeks. MGIT reached 98% recovery at 3rd week of incubation, which was faster than that of blood agar and LJ media, which needed 4 and 5 weeks to reach 98% and 96% recovery rate, respectively. Blood agar media managed to recover most of its isolates within 3rd week of incubation, a week later than MGIT but a week earlier than LJ media. This finding put blood agar media between MGIT and LJ media with time to detection. Our study showed that blood agar media can minimize the time required for growing *M.TB* on conventional LJ media by about a week.

In the current study, we managed to isolated a total of 57 mycobacterial isolates (55 were MTBC and 2 NTM) using the three isolation media. However, due to resource shortage, we had to perform DST only on blood agar and LJ media. Thus, the 47 MTBC isolates, which were positive by both LJ and blood agar media, were eligible for anti-tuberculosis DST. Of these, DST was successfully performed on 43 of the MTBC isolates.

The DST included 4 out of the 5 first line anti-tuberculosis drugs (INH, RIF, SM and EMB) excluding PZA which is not applicable on conventional solid media DST (NCCLS, 2003). Overall agreements between blood agar and LJ media were 97.7% for INH, 100% for RIF, 90.7% for SM and 97.7% for EMB. The 100% RIF and 97.7% INH agreements found in our study coincided with 100% RIF and 94.1% INH report of Coban et al (2005) and 100% for INH and RIF, 92% for SM and 96% for EMB by Coban et al (2006).

Hundred percent for sensitivity, specificity, PPV and NPV for RIF in the current study matched perfectly with other similar study (Coban *et al*, 2005). However, our study found better sensitivity (88.9%) and PPV (97.1%) for INH than the findings of Coban's group,

which was 71.4% and 93.1% sensitivity and PPV, respectively. Both studies agreed 100% regarding specificity and NPV. Another study by Coban and his group published 100% sensitivity for INH and RIF, which perfectly matches with our findings (Coban *et al.*, 2006).

In the present study relatively lower sensitivity of SM (89.2%) and EMB (96.8%) was found. Still this result is comparable with the result of a previous study, which reported sensitivity of 92% and 96% for SM and EMB, respectively (Coban *et al.*, 2006). World Health Organization, under its quality assurance program for *M.TB* DST, recently published that results for INH and RIF DST were reliable but results for SM and EMB were with some unreliability (Laszlo *et al.*, 1997). WHO suggested that unreliability can be corrected by adopting standard critical concentrations; critical proportion and reading time frame that define drug resistance.

Culture contamination rate was calculated for the three assays. Blood agar medium is prone to contamination due to the suitability of it for the growth of many fastidious microorganisms. Contamination rate was high in blood agar media when plain blood agar media was first used but with the addition of antibiotic mix, PANTA, the contamination rate was dramatically minimized. In this particular study, of all the media used blood agar was with the lowest contamination rate (5.1%) followed by LJ (9.7%) and MGIT (14.8%). Contamination rates of 1.6% in blood agar versus 7.8% in LJ media (Mathur *et al.*, 2009) and 1.5% in blood agar and BACTEC 9000 MD broth (Drancourt and Raoult, 2007) were recently reported.

A contamination rate of LJ that varied from 7.8 to 21.1% was reported in previous studies (Hanna *et al.*, 1999; Srisuwanvila *et al.*, 2008; Mathur *et al.*, 2009). In the present study, the contamination rate on LJ media was 9.7% which is better than some other studies. MGIT was the one with the highest contamination rate (14.8%). Similar contamination rate as that of ours, 15.1% in MGIT and 10.1% in LJ media was reported (Lee *et al.*, 2003). Other studies also reported from 4-17% contamination rate in MGIT (Chew *et al.*, 1998; Williams-Bouyer *et al.*, 2000; Chien *et al.*, 2000; Huang *et al.*, 2001; Oto *et al.*,

2008; Srisuwanvilai *et al.*, 2008). In general, liquid culture media are implicated in higher contamination rate than solid media due to their more enriched nature.

In the present study, blood agar media was with the lowest contamination rate followed by LJ and MGIT media. Managing contamination is a critical step when using blood agar media to grow *M.TB*, which needs prolonged incubation. The addition of PANTA to the media worked excellent and managed to reduce the contamination rate to 5.1%, which is in the acceptable range for culture contamination rate (Kent & Kubica, 1985; WHO, 1998b; CLSI, 2008). Because of the addition of PANTA, blood agar media resulted in less contamination rate than both LJ and MGIT media. However, the high cost of PANTA would make it difficult to be used for bulk and routine application. Thus, there is a need to optimize blood agar media by adding cheaper and more readily available antimicrobial agents.

8. CONCLUSIONS

In this study, blood agar media has performed as good as LJ media, which is the gold standard solid media for the isolation and DST of *M.TB*. It was even faster than LJ media in growing mycobacterial species. Furthermore, blood agar media was with the lowest contamination rate than both LJ and MGIT media. In addition to its excellent performance, it is easy to prepare with readily available ingredients, allows colony identification, cheap and safe. These qualities make blood agar media an excellent alternative for both isolation and DST of MTBC in resource limited countries like Ethiopia.

9. RECOMMENDATIONS

Firstly, this study mainly focused on the MTBC, not on other mycobacteria. Secondly, even though there were a few extra-pulmonary source specimens included in this study, the vast majority of the samples were from pulmonary source. Thirdly, in order to control contamination, an expensive antimicrobial mix, PANTA, was added on blood agar media. Thus, we recommend:

- Further studies, which focuses on extra-pulmonary TB
- Studies on *Mycobacterium* other than MTBC
- Research, which focuses on optimization of blood agar medium with cheaper antimicrobial agents to control contamination

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DECLARATION

I, the undersigned, declare that this thesis is my original work and has not been presented for a degree (or any other purpose) in any other university. All sources of materials used for this thesis have been correctly acknowledged.

Wegene Tamene Mekasha

Signature _____

Date _____